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Genomic DNA Fingerprinting by Pulsed-Field Gel Electrophoresis as an Epidemiological Marker for Study of Nosocomial Infections Caused by Methicillin-Resistant *Staphylococcus aureus*

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In this study, we have compared genomic DNA fingerprintings among isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) by using pulsed-field gel electrophoresis (PFGE). Chromosomal fragments digested with *Sma*I were most suitable for the PFGE separation. *Sma*I cut genomic DNA into 15 to 20 fragments whose sizes ranged from about 30 to 1,500 kb. Thirty-one distinctive fragment patterns were identified in 111 infecting and colonizing MRSA isolates from six different hospitals in Japan. On the basis of the genomic typing by PFGE, we performed an epidemiological investigation of an outbreak of nosocomial MRSA infections among inpatients in Nagoya University Hospital. Ten types of chromosomal digestion were identified in the 20 strains isolated from 18 infected patients and 1 from colonized hospital personnel. According to the restriction patterns, we found that four types of these strains had caused epidemic infections among 13 patients in the outbreak. Two types (types 1 and 4) of the strains were involved in the death of five patients. The other infections were sporadic. The clarity and polymorphism of the chromosomal digestion patterns enabled us to discriminate between isolates which could not be differentiated by antibiogram or plasmid analysis. Classification of the genomic DNA fingerprinting patterns by PFGE is therefore proposed as a useful method for investigating the source, transmission, and spread of nosocomial MRSA infections.

Nosocomial infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has been a major problem in large medical centers (2, 8, 11, 17, 22). This infection is sometimes life-threatening for patients with severe underlying conditions, despite extensive care. Therefore, important goals include eradicating the sources of the organisms and preventing the spread of organisms to the patients. Various markers, such as antibiograms, phage typing, and plasmid analysis, are currently used for epidemiological studies (1, 4, 5, 9, 18, 23). Antibiograms are frequently inadequate for the purpose of differentiating isolates. Phage typing has poor reproducibility and requires access to special reagents and the performance of a large number of tests. Although many investigators have reported the usefulness of plasmid analysis (1, 18, 23), this procedure has limitations. For instance, similar but not identical patterns may be observed, and isolates with only one or no plasmid cannot be reliably distinguished from similar isolates. Comparison of chromosomal digestion by using conventional electrophoresis conditions has also been performed (9, 12); however, the results show a large number of fragments that are close together on an agarose gel and may be difficult to analyze.

In view of the limitations of the markers mentioned above, a more specific methodology is needed for successful monitoring of infections. In this study, we applied the pulsed-field gel electrophoresis (PFGE) technique, which enabled us to analyze large fragments of chromosomal DNA (6, 19), to an epidemiological study of nosocomial MRSA infections. We also assessed the potential utility of this method.

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MATERIALS AND METHODS

MRSA strains. From 1989 to 1991, 111 MRSA strains (101 infecting and 10 colonizing strains) were collected: 79 from Nagoya University Hospital, 13 from hospital A, 7 from hospital B, 5 from hospital C, 4 from hospital D, and 3 from hospital E, in Aichi Prefecture, Japan. All of the strains were tested for coagulase reaction and susceptibility to methicillin or oxacillin before use in this study. The clinical sources of the infecting strains included wound swabs, pus, sputum, blood, catheters, and urine.

During this period, we encountered an outbreak of nosocomial MRSA infections in Nagoya University Hospital from February to September 1990. The sources and clinical backgrounds of the MRSA strains are listed in Table 1. Twenty strains from 18 patients and one from a doctor as a carrier were isolated. These strains were tested for chromosomal digestion patterns, antibiograms, and plasmid DNA profiles.

Chromosomal DNA analysis by PFGE. All of the 111 strains were subjected to chromosomal DNA analyses. Chromosomal DNA was prepared as described by Smith and Cantor (20). An overnight culture was harvested, washed with saline-EDTA solution (0.15 M NaCl, 10 mM EDTA [pH 8.0]), and resuspended in Pett IV solution (1 M NaCl, 10 mM EDTA [pH 8.0]). The suspension was mixed with an equal volume of 1.2% low-melting-temperature agarose (Wako Pure Chemical Co., Osaka, Japan) and allowed to solidify in a 100-μl mold. The block was incubated overnight at 37°C in a lysis solution (1 M NaCl, 0.1 M EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0], 0.5% [wt/vol] Brij 58, 0.2% [wt/vol] deoxycholate, 0.5% [wt/vol] Sarkosyl) supplemented with lysozyme (1 mg/ml [Wako Pure Chemical Co.]) and acromopeptidase (4 mg/ml [Wako Pure Chemical Co.]). The block was incubated overnight at 50°C in ES solution (0.25 M EDTA [pH 8.0], 1% [wt/vol] Sarkosyl) supplemented with

TABLE 1. Sources and clinical backgrounds of MRSA strains

Patient no.[a]	Strain no.[a]	Date of isolation (mo/day)	Ward[b]	Source	Underlying disease (date of operation [mo/day])
1	S232	5/25	ICU	Surgical wound	Aneurysm of the aorta (5/8)
2	S237	5/30	ICU	Ascites	Cancer of the bile duct (5/22)
3	S244	6/8	1B	Blood	Cancer of the pancreas (5/29)
4	S245	6/11	2B	Ascites	Malignant lymphoma, perforation of the stomach (5/30)
1	S248	6/12	1B	Surgical wound	Aneurysm of the aorta (5/8)
5	S217	2/14	ICU	Surgical wound	Abdominal rhabdomyosarcoma (2/9)
6	S239	5/30	1B	Surgical wound	Cancer of the bile duct (4/26)
7	S257	6/21	1B	Surgical wound	Esophageal cancer (4/5)
8	S252	6/13	ICU	Surgical wound	Cancer of the maxilla, perforation of the stomach (6/8)
9	S255	6/18	ICU	Surgical wound	Funnel chest (6/11)
10	S247	6/11	4A	Sputum	Acute myelocytic leukemia
9	S261	6/25	2C	Surgical wound	Funnel chest (6/11)
11	S281	7/17	2C	Skin ulcer	Ulcer of the heels
12	S330	9/4	2C	Skin erosion	Staphylococcal scalded skin syndrome
13	S331	9/5	2C	Catheter	Ileus
14	S328	9/7	2C	Catheter	Hirschsprung's disease (5/31)
15	S292	8/2	5D	Sputum	Cerebral vascular disease
16	S293	8/2	5D	Sputum	Brain tumor
17	S301	8/6	5D	Sputum	Brain tumor
18	S302	8/6	5D	Surgical wound	Cerebral aneurysm (6/21)
D[c]	S318	8/28	ICU	Nose	Carrier

[a] Strains S232 and S248 and strains S255 and S261 were isolated from the same patient (patients 1 and 9, respectively). Strain S330 was isolated from an outpatient with staphylococcal scalded skin syndrome. Thereafter, the patient entered ward 2C. Strain S318 was isolated from a doctor in the ICU.

[b] Wards 1B and 2B are surgery wards; ward 4A is a medical ward; ward 2C is a pediatric surgery ward; and ward 5D is a neurosurgery ward.

[c] D, a doctor.

proteinase K (0.1 mg/ml [Wako Pure Chemical Co.]) and was then treated with 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) for 4 h and washed four times with TE buffer. Thinly sliced sections of block (about 10 μl) were digested with 10 U of *Kpn*I, *Eco*RI, *Xho*I, *Bam*HI, *Sma*I, and *Not*I (Takara Shuzo Co., Kyoto, Japan) for 18 h and then electrophoresed through a 0.9% agarose gel in TBE buffer (0.1 M Tris, 0.1 M boric acid, 2 mM EDTA [pH 8.0]) at 10°C by using the contour-clamped homogeneous electric field (CHEF) system (Pulsaphor Plus; Pharmacia LKB Biotechnology, Uppsala, Sweden). The conditions for electrophoresis were 200 V for 15 h, with pulse times ranging from 5 to 45 s for *Kpn*I, *Eco*RI, *Xho*I, and *Bam*HI digestions, and 170 V for 30 h, with pulse times ranging from 10 to 100 s for *Sma*I and *Not*I digestions. Thereafter, the gels were stained with ethidium bromide, washed with distilled water, and photographed. *Saccharomyces cerevisiae* genomes or lambda DNA concatemers (Bio-Rad Laboratories, Richmond, Calif.), or both, were used as the size standard.

Antibiogram. Twenty strains from the 18 patients with MRSA infections contracted in the outbreak (Table 1) were tested for antibiotic resistance. The susceptibilities to antimicrobial agents except for rifampin were determined by the standard disk method (15). The antibiotic disks used in this study were penicillin G, 10 IU; methicillin, 5 μg; cefazolin, 30 μg; cefmetazole, 30 μg; clavulanic acid/amoxicillin, 10/20 μg; gentamicin, 10 μg; minocycline, 30 μg; and erythromycin, 15 μg. The susceptibility to rifampin was determined by the agar dilution method (16).

Plasmid DNA analysis. Plasmid DNA from 11 strains isolated from patients 1 through 10 (Table 1) was extracted by the modified alkaline lysis method (3). An overnight culture was harvested, washed with saline-EDTA solution (0.15 M NaCl, 10 mM EDTA [pH 8.0]), resuspended in 0.1 ml of glucose-TE buffer (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0]) containing achromopeptidase (1 mg/ml; Wako Pure Chemical Co.), and incubated for 30 min at 37°C. To this, 0.2 ml of 0.2 M NaOH–1% (wt/vol) sodium dodecyl sulfate was added, and the mixture was allowed to stand on ice for 10 min; then 0.15 ml of 3 M potassium–5 M acetate was added, and the mixture was cooled for another 10 min on ice. The mixture was centrifuged at 12,000 × *g* for 5 min, and the supernatant containing plasmid DNA was extracted once with buffer-saturated phenol mixture. The DNA was electrophoresed through a 0.9% agarose gel, stained with ethidium bromide, and photographed. Lambda/*Hin*dIII DNA was used as the size standard.

RESULTS

Chromosomal DNA analysis by PFGE. We screened various restriction enzymes to see which would cut the chromosomal DNA into a convenient number of fragments. *Sma*I cut the chromosomal DNA into 15 to 20 fragments ranging from 30 to 1,500 kb. *Kpn*I, *Eco*RI, *Xho*I, and *Bam*HI cut it into a large number of fragments smaller than 50 kb, and *Not*I cut it into only three fragments (data not shown). Therefore, *Sma*I was chosen for differentiating isolates in this study.

Thirty-one distinctive restriction patterns were identified in the 111 MRSA strains tested (Fig. 1). Types 1 through 5 were frequently observed in strains from infected patients in Nagoya University Hospital. Type 3 was also frequently identified in hospital A. Ten colonizing strains were of the following types: type 1 (one strain), type 2 (one strain), type 3 (two strains), type 4 type 6, type 13, type 17, type 19, and type 24 (one strain each). The type distribution of the strains was not different among the infected sites. We observed that the restriction patterns of the strains isolated successively from 9 individual patients remained unchanged (data not shown).

As a result of the rich diversity of the chromosomal

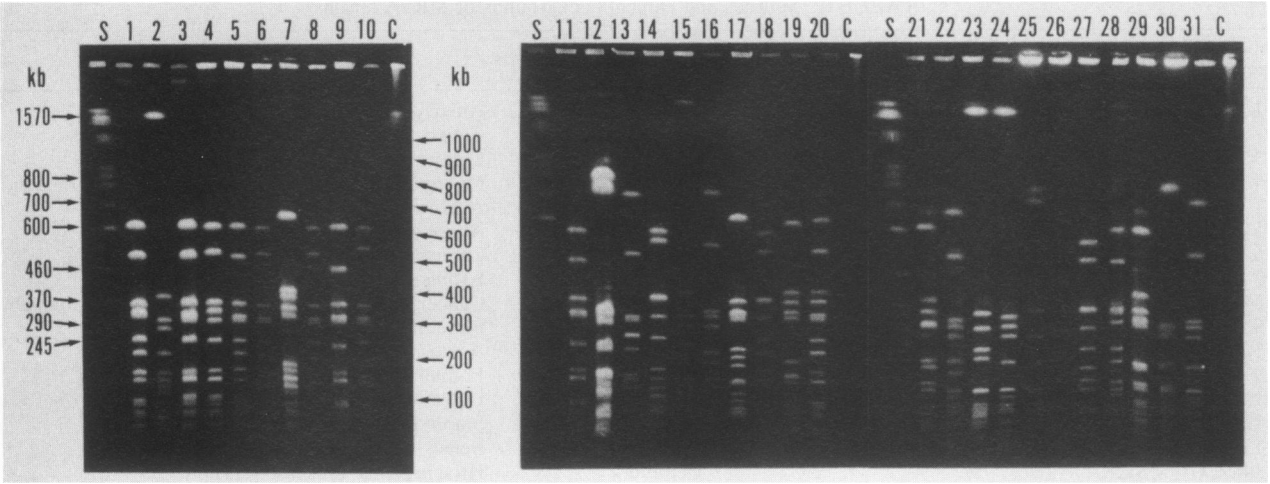

FIG. 1. PFGE separation of restriction fragments of the MRSA genome digested with *Sma*I. A total of 111 MRSA strains from six different hospitals were tested for chromosomal digestion. Lanes 1 through 31 represent type numbers. Types 1 through 16 contained the following numbers of MRSA strains, respectively: 15, 15, 13, 10, 6, 6, 6, 4, 4, 3, 3, 3, 2, 2, 2, and 2. Types 17 through 31 contained only one strain each.

digestion profiles, we used this method in a epidemiological study of an outbreak of nosocomial MRSA infection among inpatients of Nagoya University Hospital from February to September 1990. Ten types of restriction patterns were found in the 21 strains tested (Fig. 2; Table 2). Although the restriction profiles of S245 and S331, whose chromosomal patterns were types 1 and 6, respectively, showed a very close resemblance, minor differences between them were evident on a single gel (Fig. 2). Figure 3 shows the temporal relationships among 13 patients with MRSA infections in the outbreak. Four types (types 1, 3, 4, and 5) of MRSA strains infected 13 patients in the intensive care unit (ICU), surgery wards, pediatric surgery ward, and neurosurgery ward (Table 2). The type 1 strain infected patients 1 through 4; the type 3 strain infected patients 5 through 7; the type 4 strain infected patients 8, 17, and 18; and the type 5 strain infected patients 11, 13, and 15. These four strains might have been transmitted between the patients or between hospital personnel and the patients. Thus, these infections were considered to be epidemic. Five patients (patients 2, 3, 4, 8, and 17) died of a combination of MRSA infections and severe underlying diseases. The type 1 and 4 strains caused these life-threatening infections. The other infections, caused by type 6, 8, 10, 13, and 14 strains, were considered to be sporadic.

Antibiogram. The antibiograms are shown in Table 2. Except for S330, the MRSA strains tested were highly resistant to penicillin G, methicillin, cefazolin, cefmetazole, clavulanic acid/amoxicillin, and erythromycin. The susceptibilities to the other antibiotics varied among strains. The resistance to rifampin was characteristic in strains S217, S239, and S257. Accordingly, five types of antibiotic patterns were exhibited (Table 2).

Plasmid DNA analysis. Figure 4 shows the plasmid pattern of each strain. Six strains (S232, S237, S244, S245, S248, and S247) contained three plasmids of 3.4, 1.9, and 1.6 kb. Strain S255 contained three plasmids of 2.8, 1.7, and 1.5 kb. The remaining four strains (S217, S239, S257, and S252) con-

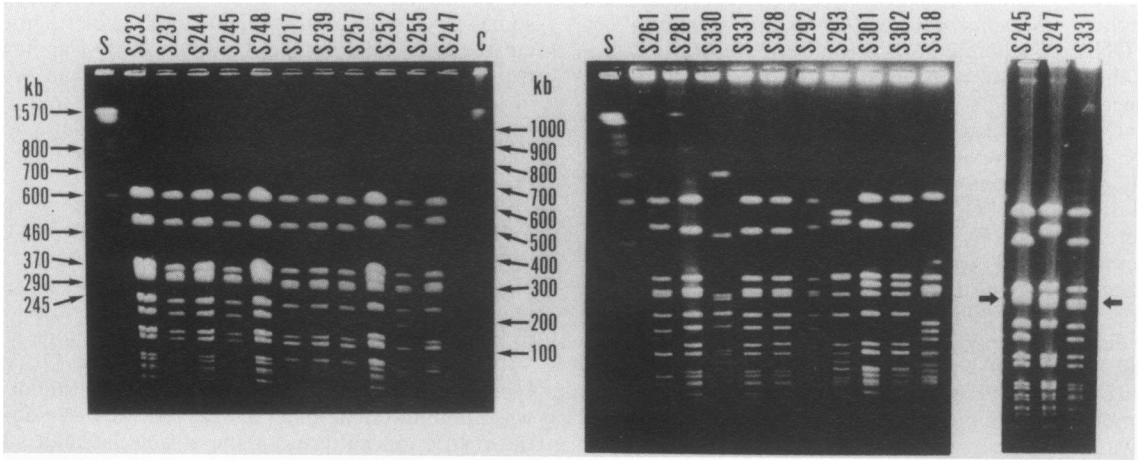

FIG. 2. Chromosomal digestion patterns of 21 MRSA isolates from 18 patients with MRSA infections and one member of the hospital staff with MRSA colonization in an outbreak of nosocomial MRSA infections. Lanes S and C contain *S. cerevisiae* genomes and lambda DNA concatemers, respectively, as size markers. Two arrows show the difference between the patterns of S245 and S331.

TABLE 2. Classification of MRSA strains on the basis of antibiotic, chromosomal, and plasmid patterns

Patient no.	Strain no.	Ward	Antibiogram[a]									Antibiotic pattern	Chromosomal pattern	Plasmid pattern
			Pcg	Dmp	Cez	Cmz	Cva	Gm	Min	Em	Rfp			
1	S232	ICU	R	R	R	R	R	S	R	R	S	A-1	C-1	P-1
2	S237	ICU	R	R	R	R	R	S	I	R	S	A-1	C-1	P-1
3	S244	1B	R	R	R	R	R	S	I	R	S	A-1	C-1	P-1
4	S245	2B	R	R	R	R	R	S	R	R	S	A-1	C-1	P-1
1	S248	1B	R	R	R	R	R	S	I	R	S	A-1	C-1	P-1
5	S217	ICU	R	R	R	R	R	R	I	R	R	A-2	C-3	P-2
6	S239	1B	R	R	R	R	R	R	S	R	R	A-2	C-3	P-2
7	S257	1B	R	R	R	R	R	R	S	R	R	A-2	C-3	P-2
8	S252	ICU	R	R	R	R	R	R	S	R	S	A-3	C-4	P-2
9	S255	ICU	R	R	R	R	R	S	S	R	S	A-4	C-8	P-3
10	S247	4A	R	R	R	R	R	R	S	R	S	A-3	C-10	P-1
9	S261	2C	R	R	R	R	R	S	S	R	S	A-4	C-8	NT[b]
11	S281	2C	R	R	R	R	R	R	S	R	S	A-3	C-5	NT
12	S330	2C	R	R	S	S	R	S	S	R	S	A-5	C-13	NT
13	S331	2C	R	R	R	R	R	R	S	R	S	A-3	C-5	NT
14	S328	2C	R	R	R	R	R	R	S	R	S	A-3	C-5	NT
15	S292	5D	R	R	R	R	R	R	S	R	S	A-3	C-6	NT
16	S293	5D	R	R	R	R	R	R	S	R	S	A-3	C-14	NT
17	S301	5D	R	R	R	R	R	R	S	R	S	A-3	C-4	NT
18	S302	5D	R	R	R	R	R	R	S	R	S	A-3	C-4	NT
D	S318	ICU					NT						C-17	NT

[a] Abbreviations: Pcg, penicillin G; Dmp, methicillin; Cez, cefazolin; Cmz, cefmetazole; Cva, clavulanic acid/amoxicillin; Gm, gentamicin; Min, minocycline; Em, erythromycin; Rfp, rifampin; R, resistant; S, susceptible; I, intermediate.
[b] NT, not tested.

tained none of these plasmids. Although large plasmids (>20 kb) might be carried by some strains, the differences between them could not be clearly identified by electrophoresis. Accordingly, three types of plasmid patterns were observed (Table 2).

DISCUSSION

The reliability of markers for epidemiological investigations of nosocomial infections depends on diversity and reproducibility. Genetic techniques are currently used to compare isolates. Recent reports indicate that plasmid analysis is also useful (1, 23). Digestion of total cellular DNA with restriction enzymes is also reported to be useful (9, 12); however, under conventional electrophoretic conditions, the results may be difficult to analyze.

Recently, the PFGE technique has been used to separate large fragments of bacterial DNA (6, 7, 14, 19–21), and PFGE has effectively separated yeast chromosomal DNA molecules up to 2,000 kb (19). PFGE has been used to construct a physical map of the *Escherichia coli* K-12 genome (20). Moreover, comparison of the genomic DNAs of various enterococcal isolates by using restriction endonucleases showed the potential utility of this procedure for epidemiological studies (14). The choice of the restriction endonucleases depends on the G+C content of the organism to be tested as well as on the recognition sequence of the enzyme. Since the G+C content of *S. aureus* is 32 to 36% (10), restriction enzymes with recognition sequences that contain only G and C, such as *Sma*I (CCCGGG) and *Not*I (GCGGCCGC), are expected to generate only a limited number of large fragments. As expected, *Sma*I gave only 15 to 20 fragments whose size distributions were appropriate for the PFGE separation (Fig. 1). Our results show that the *Sma*I digestion is most suitable for comparing genomic DNAs of MRSA isolates.

In this study, we applied this procedure to the epidemiological investigation of nosocomial MRSA infections. First, we extended the evaluation of chromosomal digestion patterns by characterizing MRSA strains in hospital settings. We tested a large number of MRSA strains for chromosomal digestion and compared their fragment patterns. The assignment of an individual isolate to a particular pattern was verified by direct comparison. When strains analyzed on different gels appeared to have similar restriction patterns, they were run together on a single gel to clarify their relationship. Next, we applied the fragment patterns to an epidemiological investigation of nosocomial MRSA infections and evaluated the utility of this method.

According to the *Sma*I digestion, 31 types of restriction patterns were exhibited in 111 MRSA strains tested (Fig. 1). The diversity of this method was similar to that of restriction enzyme profiles of plasmid DNA. Earlier investigators observed 37 different *Eco*RI fragment profiles of plasmid DNA in 120 independently isolated MRSA strains, and they revealed the potential utility of endonuclease digestion profiles of plasmid DNA for epidemiological studies (23). In the present study, however, comparisons of the potential utilities of these two methods are difficult, since the number of MRSA strains tested for plasmid profiles was small and the plasmid DNAs were not digested with restriction endonuclease. Plasmid digestion profiles are useful when the bacteria contain one or more plasmids, the plasmid composition of the strain remains stable throughout the infection, and the differences between plasmids are distinguishable by the digested fragments. Chromosomal digestion profiles, on the other hand, are distinguishable regardless of these factors. In our study, 4 (36%) of the 11 MRSA strains tested contained no distinctive plasmid. Therefore, we consider that chromosomal fingerprinting with PFGE is an effective epidemiological marker.

In this study, we have established the temporal relationships among 13 patients with MRSA infections that were contracted during an outbreak (Fig. 3). Interestingly, of the

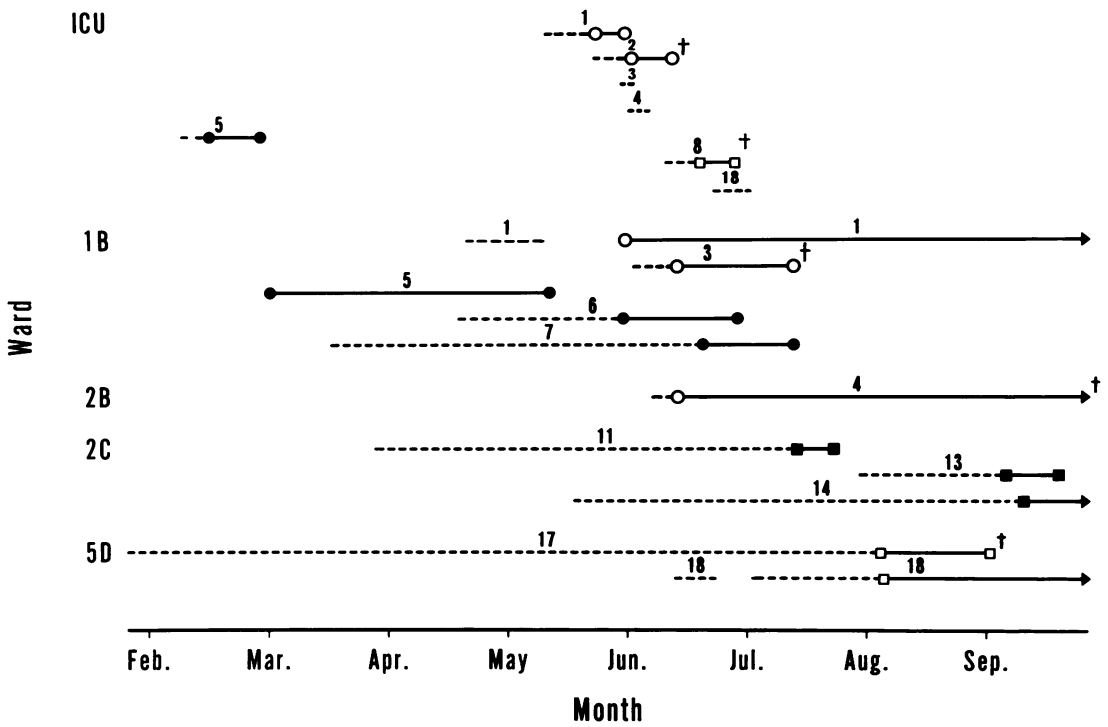

FIG. 3. Temporal relationship among 13 patients with epidemic MRSA infections occurring in an outbreak. Patients 2, 3, 4, 8, and 17 died of a combination of MRSA infections and severe underlying diseases. Patient 4 died in February 1991. Symbols: – – –, hospitalization in each ward; ——, presence of the MRSA strains in specimens; →, hospitalization after the time of the outbreak; ○, type 1; ●, type 3; □, type 4; ■, type 5; †, patient's death.

four types of epidemic strains, type 1 and 4 strains in particular caused life-threatening infections. Five patients died of severe infections with these types of MRSA strains (in combination with their underlying condition), so we should continue monitoring for these types especially. During the outbreak, we screened ICU personnel and environmental surfaces for MRSA to detect the sources of these epidemic strains. Although a doctor in the ICU was identified as a carrier, the genomic DNA fingerprinting of the isolate was different from those of the infecting strains. However, after the outbreak, the type 1 strain was isolated from six infected patients on various wards. There is extensive movement of surgeons, physicians, and other hospital personnel between the ICU and other wards, especially between the ICU and the surgery wards, while caring for postoperative patients. Thus, transient carriage on hospital personnel might be an important way in which MRSA infection is spread. Attempts to prevent transmission of these epidemic infections should include isolating infected patients and thorough hand-washing and gown techniques for hospital personnel, especially while caring for postoperative patients.

As well as epidemic infections, sporadic or endogenous infections were identified in five patients in the outbreak (patients 9, 10, 12, 15, and 16). We also observed that colonizing and infecting strains had the same restriction pattern in two individual patients in the survey of the 111 MRSA strains. These observations suggest that nosocomial MRSA infections may arise in part from endogenously carried strains. A recent report indicates that colonization of the anterior nares by MRSA predicts the development of MRSA infection in long-term-care patients (13). Therefore, eliminating the carrier state, particularly in postoperative patients, should be required.

This study showed a considerable diversity in the chromosomal digestion patterns of MRSA isolates. The chromosomal digestion patterns permitted us to clearly differentiate the MRSA isolates, even those showing the same antibiogram or the same plasmid profile. Our study also demonstrated a reproducibility in the chromosomal digestion patterns from MRSA isolates. The chromosomal patterns of the MRSA strains isolated repeatedly from the same patient remained unchanged (Table 2). Because of the diversity and reproducibility, the classification of genomic DNA finger-

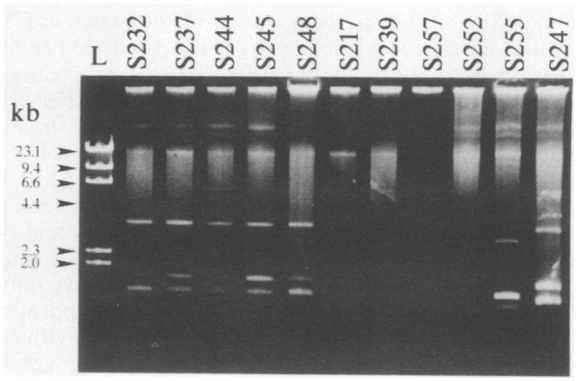

FIG. 4. Gel electrophoresis of plasmid DNA from MRSA strains. Lane L contains marker DNA fragments (lambda/*Hin*dIII).

printing patterns by PFGE is proposed as an effective means of differentiating among MRSA strains.

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